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Falsely low phosphatidylethanol may be associated with biomarkers of haemolytic disease

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Abstract

Aims: Falsely lower or even negative phosphatidylethanol (PEth) levels may theoretically be seen in patients with haemolytic diseases, and the present study aimed to elucidate this hypothesis.

Methods: PEth and carbohydrate-deficient transferrin (CDT) from 9893 serum and whole blood samples were included along with markers of haemolysis (i.e. haptoglobin, HbA1c, reticulocytes, LD and Hb). Cases showing discrepancy between PEth and CDT, that is, a low PEth value and a high CDT value, were considered to be possibly caused by falsely lowered PEth despite high alcohol consumption. These cases ($N = 233$) were compared to the control group without PEth and CDT mismatch.

Results: The levels of haptoglobin were significantly lower in the cases showing low PEth and high CDT (estimate $= -0.62$, $p = 0.002$). The levels of HbA1c (estimate $= -3.26$, $p = 0.001$) and Hb (estimate $= -0.507$, $p < 0.001$) were also significantly lower in this group. These findings indicate haemolytic diseases in the low PEth/high CDT group. There were no significant differences for reticulocytes and LD concentrations between the low PEth/high CDT group and the control group.

Conclusions: These results indicate that falsely low PEth values could be associated with markers of haemolytic diseases, although more research is needed to highlight this further.

KEYWORDS
alcohol biomarker, carbohydrate-deficient transferrin, haptoglobin, haemolytic diseases, phosphatidylethanol

1 | INTRODUCTION

Phosphatidylethanol (PEth) has become a widely used biomarker for detecting high intake of alcohol, and it is a useful tool for healthcare professionals in assessing harmful alcohol consumption in patients with a variety of diseases and conditions. Advantages of PEth include high sensitivity and specificity, in addition to ability to

distinguish between moderate and heavy alcohol consumption.[1–5]

Seen in the context of reported alcohol consumption, PEth formation has shown significant variation between individuals.[6] PEth is a group of phospholipids formed by the enzyme phospholipase D (PLD) in the cell membrane of the erythrocytes as direct metabolites of alcohol.[7] Thus, PEth is an example of a direct alcohol marker, which can be measured in blood only after intake of ethanol and is less affected by medical conditions, as could be the case for indirect alcohol biomarkers.[8] PEth 16:0/18:1 is the dominant homologue of the phosphatidylethanols and is regularly used in laboratory analyses.[9,10]

Carbohydrate-deficient transferrin (CDT) is a more traditional, more studied and widely used biomarker of alcohol abuse. However, a body of evidence point out that PEth is more sensitive than CDT.[1,4,11,12] As serum CDT originates in the liver whereas clinically measured PEth is harboured in erythrocytes, it is naturally to assume that erythrocyte lifespan may affect levels of PEth. Other biomarkers associated with the components of the erythrocytes are affected by the erythrocyte lifespan—for example, a glycated form of the haemoglobin (HbA1c) has previously been shown to be falsely low due to haemolytic diseases.[13,14] One could thus hypothesise that PEth, which is bound to the cell membrane of the red blood cells, also would be affected by a high erythrocyte turnover due to in vivo haemolytic diseases and conditions, as destruction of red blood cells would be expected to remove PEth from the circulation.[15] On the other hand, in vitro haemolysis after sample collection would not affect the levels of PEth, as PEth would still be present in the sample. In order to elucidate this hypothesis, it is necessary to detect 'heavy drinkers' with possibly falsely low PEth values. This could be the case in patients showing a combination of high values of CDT and low values of PEth.

Haemolysis cause shortening of erythrocyte lifespan and includes a wide range of pathophysiological conditions, with several diagnostic biomarkers available. Haemoglobin (Hb) levels are frequently analysed. In addition, evidence of haemolysis includes increased lactate dehydrogenase (LD), low haptoglobin, increased reticulocyte count and increased bilirubin.[16]

Haptoglobin is significantly reduced in both intravascular and extravascular forms of haemolysis by binding free circulating haemoglobin in the blood stream.[17] Low or unmeasurable level of haptoglobin in a blood sample is highly diagnostic for haemolytic diseases.[16]

Reticulocytes are precursors of red blood cells, and in some haemolytic diseases, an increased number are released from the bone marrow and can be measured in the bloodstream.[16]

LD is not a biomarker specific to haemolysis.[16] It is often slightly increased in the blood stream in extravascular haemolytic conditions, whereas the levels rise more in intravascular haemolysis.[18]

Thus, haemolytic diseases could be suspected in a patient with a low Hb, a low level of haptoglobin and high levels of reticulocytes and LD. In addition, levels of HbA1c below the reference range could also be measured as a result of haemolytic diseases.

In the present study, we aimed to explore a possible association between biomarkers suggesting haemolytic diseases and falsely low values of PEth 16:0/18:1. LD, haptoglobin, reticulocytes, HbA1c and Hb were included in the study, together with PEth and CDT. A representative patient case with repeated measurements of relevant biomarkers is also described.

2 | MATERIAL AND METHODS

2.1 | Data collection

Results from PEth, CDT, haptoglobin, reticulocytes, LD, HbA1c and Hb analyses performed over the period from September 2016 to January 2021 at the Fürst Medisinsk Laboratorium were used for the present study. The study database contained anonymous and encrypted information on age and sex in addition to sampling dates and analytical results. Samples were mostly collected from patients of primary care physicians in addition to some from social care institutions. However, further information about the study population could not be obtained, and for ethical reasons, no medical diagnoses or other case history from the patients was available. Patients were included if both PEth and CDT were measured simultaneously (PEth in whole blood and CDT in serum), together with one or more of the analytes haptoglobin, reticulocytes, LD and HbA1c. Hb results were also included in the study. It should be noted that all analyses were routine requests and no extra analyses were performed in conjunction with this study. Additional analyses were impossible as the blood and serum samples were discarded after all routine analyses were performed.

2.2 | Sample preparation

Serum and EDTA-anticoagulated whole blood samples were obtained by drawing venous blood into the serum SST or EDTA-prefilled vacutainer tubes, respectively (both from Becton Dickinson Norway, Oslo, Norway), with further processing according to manufacturer's

instructions. All analytes were measured within the same day after sampling upon arrival to the laboratory.

2.3 | Analysis of PEth and CDT

PEth and CDT were analysed as described in detail in a previous publication.[11] In brief, PEth was analysed in whole blood using a Waters Acquity UPC2 (TM) UltraPerformance Convergence chromatography system connected to Waters TQ-S triple quadrupole mass spectrometer (UPC2-MS/MS) (Waters, Milford, MA, USA). Serum carbohydrate-deficient transferrin (CDT) was quantified by electrophoretic separation of the transferrin fractions using a classic Sebia Capillarys 2 (Lisses, France) without CDT-IFCC standardisation. The limit of quantification was 0.015 μmol/L (10.5 ng/ml) for PEth and 0.4% units for CDT. PEth values below 0.03 μmol/L were reported as negative.

PEth and CDT were related to alcohol consumption according to previously published results and clinical practice. Values of PEth > 0.30 μmol/l[9] and CDT ≥ 1.7% units[19] were considered to represent harmful alcohol consumption. PEth levels of 0.30 μmol/L correspond to approximately 210 ng/ml (exactly 210.9 ng/ml), but as the limit of 0.30 μmol/L is commonly reported,[5] this unit is further used in the present article.

2.4 | Analysis of haptoglobin, reticulocytes, LD, HbA1c and Hb

Serum haptoglobin and LD were analysed using Siemens ADVIA Chemistry XPT system (Siemens Healthcare AS, Oslo, Norway). Blood HbA1c levels were measured in the lysates of EDTA-anticoagulated whole blood using Tosoh HLC723-G8 automated glycohaemoglobin analyser (Sysmex Norway NUF, Skjetten, Norway). Levels of Hb and reticulocyte counts were determined using Sysmex XN-9100 haematology analyser (Sysmex Norway NUF, Skjetten, Norway).

2.5 | Study group and control group

In order to investigate a possible connection between haemolysis and falsely low PEth values, we divided our data material into a study group and a control group. The study group included the cases where the measured PEth value indicated a non-harmful use of alcohol (≤0.30 μmol/L), whereas the CDT value at the same time was in accordance with harmful alcohol consumption (≥1.7% units). This group is referred to as low PEth/high CDT.

The remaining cases showed an expected relation between PEth and CDT levels. This included high CDT/high PEth or low CDT/low PEth. Also, as PEth is documented to be more sensitive than CDT,[11] the combination of high PEth/low CDT was also considered to represent an expected relation between PEth and CDT. All these combinations of PEth and CDT constituted the control group.

2.6 | Statistics

SPSS IBM SPSS® Software version 26.0 was used for statistic evaluation of the data.

Linear mixed model analyses (using random intercept and the restricted maximum likelihood model) were used to allow for inclusion of multiple samples per patient. Five separate linear mixed model analyses were performed using haptoglobin, LD, reticulocytes, HbA1c and Hb, respectively, as the dependent variable. The presence of low PEth levels combined with high CDT levels was inserted to the model as fixed effects together with age and sex (main effects, Type III). Estimates from the mixed model are presented together with 95% confidence intervals and *p*-values for different concentrations of haptoglobin, LD, reticulocytes, HbA1c and Hb in the study group compared to the control group.

2.7 | Patient case

As an example, we present a patient with five blood samples, with detailed levels of PEth and CDT compared to haptoglobin, HbA1c, reticulocytes and LD concentrations.

2.8 | Ethics

Ethical approval was obtained from Regional Committee for Medical and Health Research Ethics, Region South East, Norway (2018/1041). Due to the large size of the data material and the anonymous handling of the data, the study was approved to be performed without informed consent from each of the participants. The study was conducted in accordance with the *Basic & Clinical Pharmacology & Toxicology* policy for experimental and clinical studies.[20]

3 | RESULTS

Five thousand seven hundred and forty-six patients had results of both PEth and CDT in addition to at least one of the analytes haptoglobin, reticulocytes, LD or HbA1c

measured from the same blood sampling occasion. Hb was frequently measured, and these results were also included. The patient cohort consisted of 66% males and 34% females. Their ages ranged from 18 to 99 years with a median of 56. Half of the patients were between 45.5 and 66.5 years old [interquartile range (IQR) of 21]. The number of samples per patient ranged from 1 to 50. However, most patients had only one sample (median value was one). In total, there were 9893 included samples.

The median PEth concentration for all samples was 0.23 μmol/L (IQR 0.72), and 4361 (44%) of the samples had a PEth value >0.30 μmol/L, suggesting a high alcohol intake. In comparison, 2324 (23.5%) of the CDT values would indicate a high consumption (≥1.7% units). For all samples, the median CDT concentration was 0.9 (IQR 0.9).

Of the total 9893 samples, 233 (2.4%) had a low PEth value (≤0.30 μmol/L) and simultaneously a high CDT value (≥1.7% units). These samples make up the study group, referred to as low PEth/high CDT, and they were taken from 173 different patients. The remaining 9660 samples (from 5612 patients) showed an expected relation between PEth and CDT results and constitute the control group. A few number of patients had samples included in both the study group and the control group. A flow chart of the included cases is seen in Figure 1. The median ratio between PEth and CDT was 0.079 (IQR 0.06) in the study group and 0.20 (IQR 0.43) in the control group. Ethanol was measured in 1228 of the samples, and the level was at or above 0.5 g/L in 94 of these.

The number of samples with valid results for the study group and the control group as well as median concentrations for haptoglobin, HbA1c, LD, reticulocytes and Hb are shown in Table 1.

Figure 2 shows the haptoglobin concentrations for all samples in the study group compared to the control group. The median concentration of haptoglobin among all samples was 1.3 g/L (IQR 0.7) in the control group and 0.6 g/L (IQR 0.7) in the study group.

In the linear mixed model, correcting for age and sex, there were significantly lower concentrations of haptoglobin ($p = 0.002$) in the patients showing a combination of low PEth and high CDT compared to the control group. The estimate was −0.62 g/l, that is, the concentrations of haptoglobin was 0.62 g/L lower in the study group (low PEth/high CDT) compared to the control group. There were also significantly lower concentrations of HbA1c (estimate = −3.26 mmol/mol, $p = 0.001$) and Hb (estimate = −0.507 g/dl, $p < 0.001$) in the study group compared to the control group. However, for reticulocytes and LD, there was no significant difference in concentrations between the study group and the control group ($p = 0.422$ and $p = 0.574$, respectively). This is seen in Table 2. In addition, all statistical analyses were also performed using logarithmically transformed values and yielding similar results.

3.1 | Patient case

A male patient in his fifties had five samples taken during about 2 years' time, where PEth and CDT were measured together with reticulocytes, haptoglobin or HbA1c. These results are presented in detail in Table 3. All samples showed a low PEth value combined with a high CDT value. In all measured samples, haemolysis was also indicated, as the concentration of haptoglobin was below the limit of quantification, HbA1c was below the reference range, and the concentrations of reticulocytes were above the reference range in all measured samples. LD was mostly above the reference range. The GGT and ferritin values indicated a high alcohol consumption despite low PEth values.

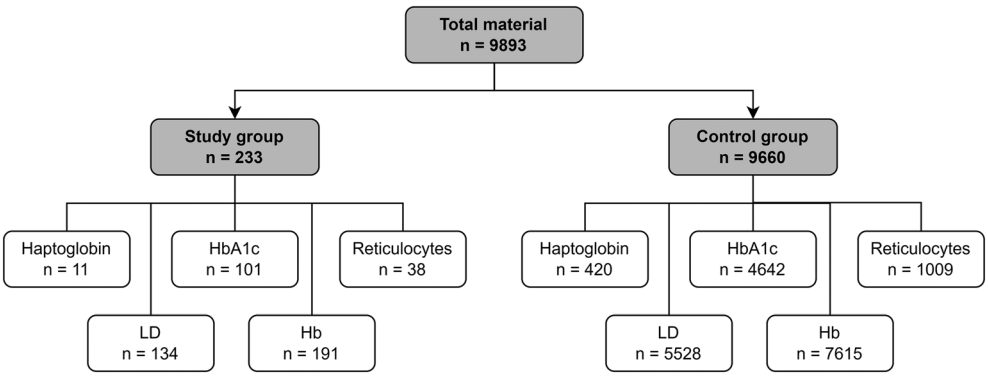

FIGURE 1 A flow chart showing the included cases in the study group and the control group, respectively. Please note that more than one of the markers haptoglobin, HbA1c, LD, reticulocytes and Hb were analysed in many samples; therefore, the sum exceeds the number of samples in the study group and control group, respectively.

TABLE 1 Number of samples in the study and control group, and median values of total samples for haptoglobin, HbA1c, LD, reticulocytes and Hb

	Haptoglobin	HbA1c	LD	Reticulocytes	Hb
No. samples study group	11	101	134	38	191
No. samples control group	420	4642	5528	1009	7615
Median all samples (IQR)	1.3 (0.7)	38 (7)	192 (45)	0.07 (0.02)	Total: 14.5 (2.0) Male: 15.0 (1.8) Female: 13.7 (1.6)
Reference range	0.4–2.1	28–42	<205 (<70 years) <255 (≥70 years)	0.02–0.08	Male: 13.4–17.0 Female: 11.7–15.3

Notes: Units for median values and reference ranges: haptoglobin (g/L), HbA1c (mmol/mol), LD (U/L), reticulocytes ($\times 10^{12}$) and Hb (g/dL). PEth and CDT were measured in all samples.
Abbreviation: IQR, interquartile range.

FIGURE 2 Concentrations of haptoglobin in the low PEth/high CDT group and in the control group. The box length is the interquartile range (25th–75th percentile) of the concentrations. The line across the inside of the box represents the median value. Whiskers represent the largest or smallest value within 1.5 times the interquartile range. Circles represent values exceeding 1.5 times the interquartile range.

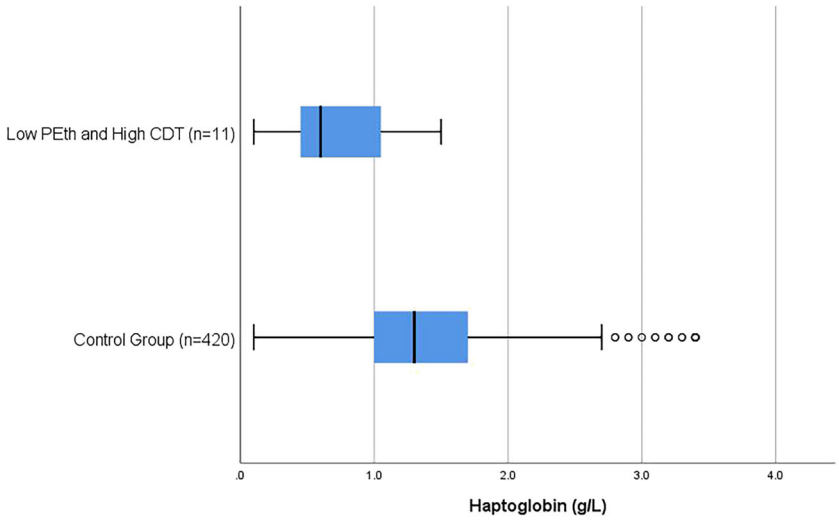

TABLE 2 Estimates with 95% confidence interval (CI) from the linear mixed model for the difference in haptoglobin, LD, reticulocytes, HbA1c and Hb between the study group and the control group

Variable	Estimate	Lower 95% CI	Upper 95% CI	*p*-value	*N*
Haptoglobin	−0.62	−1.01	−0.23	0.002	431
LD	−3.99	−17.9	9.9	0.574	5662
Reticulocytes	0.0035	−0.0050	0.012	0.422	1047
HbA1c	−3.26	−5.18	−1.33	0.001	4743
Hb	−0.507	−0.709	−0.306	<0.001	7806

Notes: The *p*-values show the significance for the differences between the study group (low PEth/high CDT) and the control group. Units for estimates: haptoglobin (g/L), HbA1c (mmol/mol), LD (U/L), reticulocytes ($\times 10^{12}$) and Hb (g/dL).

4 | DISCUSSION

This study investigated patients with a combination of low PEth and high CDT and thereby possibly falsely lowered PEth values. These patients had lower concentrations of haptoglobin, HbA1c and Hb, compared to patients showing an expected relation between CDT and PEth. A condition of haemolysis could therefore be suspected. However, no differences were seen for the concentrations of reticulocytes and LD.

The results of this study indicate a possible association between falsely low PEth values and haemolytic diseases, which requires further investigations. Observation of low haptoglobin levels in the group of patients showing high CDT combined with low PEth is suggestive of haemolysis as a mechanism for falsely lowered PEth.

TABLE 3 Patient case with five samples and detailed levels of CDT, PEth, haptoglobin, reticulocytes, HbA1c, LD, GGT and ferritin

Sample number	CDT	PEth	Haptoglobin (0.4–2.10 g/L)	Reticulocytes (0.02–0.08 × 10^{12}/L)	HbA1c (28–42 mmol/mol)	LD (<205 U/l)	GGT (<115 U/L)	Ferritin (20–300 µg/L)
1	9.5	0.25	-	-	14	186	146	352
2	2.9	<0.03	< 0.1	0.15	-	232	131	330
3	2.6	<0.03	< 0.1	0.17	-		155	293
4	6.3	0.15	-	0.13	-	217	156	466
5	3.3	0.06	-	0.11	25	214	148	-

Note: Reference ranges (used by Scandinavian laboratories) for the biomarkers in parentheses.

Although theoretically CDT could be by chance just above the reference range while PEth is slightly below, the ratio between PEth and CDT was highly different in the study group and the control group. Our data also showed that the median PEth/CDT ratio was more than twice as high for the samples with haptoglobin within the reference range (0.4–2.10 g/L) ($n = 366$) than in the samples where haptoglobin was below the reference range and haemolytic diseases therefore was indicated ($n = 20$). This further strenghtens the assumption that PEth is actually lowered compared to CDT in the patients showing low levels of haptoglobin as a result of haemolytic diseases.

Alterations in levels of Hb and its glycosilated form HbA1c are well described in haemolytic diseases.[21] Although HbA1c is not a marker of haemolysis, it is well known to be falsely lowered during haemolytic conditions,[13,14] and the present study indicates that the situation is mechanistically similar for HbA1c and PEth. Reduced Hb levels are only detected in more severe cases of haemolysis, and normal or near-normal levels can be seen in mild forms of haemolysis.[18] The finding of lower Hb in the low PEth/high CDT group therefore also adds to the indication of haemolytic conditions present in this group.

On the other hand, we found no differences in reticulocytes and LD between the two groups that could strengthen our hypothesis even further. However, an increased production of reticulocytes, as precursors of new red blood cells, requires a proper bone marrow response to haemolysis providing adequate levels of iron, folate and vitamin B12 as well as preserved secretion of erythropoietin.[16] This means that patients with concomitant conditions such as malabsorption, malnutrition, bone marrow abnormalities or chronic kidney disease may exhibit low or normal reticulocyte counts despite haemolysis. When it comes to LD, significantly increased levels can be seen in the bloodstream especially in some types of haemolysis, but not necessarily in all cases.[18] Also, LD is an unspecific enzyme, which could potentially be elevated in patients in the much bigger control group due to other conditions than haemolysis and thus mask possible effects of haemolytic conditions.

The balance between synthesis rate and elimination rate determines the measured PEth concentration. A previous in vitro study, which investigated factors affecting PEth formation, found no significant correlation with the haematology parameters Hb, erythrocyte volume fraction (EVF), red blood cell count (RBC) or mean corpuscular volume (MCV).[6] We would thus not expect haemolytic diseases to affect the synthesis of PEth per se. However, phosphatidylethanols are constituents of the erythrocyte cell membrane, and haemolytic conditions, which cause red blood cells to be eliminated from the blood circulation at a higher rate than normal, could therefore lead to lower PEth values than expected from a high alcohol consumption.[15]

In the presented patient case, all the CDT values were clearly above 1.7% units and indicate a high alcohol consumption despite low PEth values. GGT and ferritin concentrations above the reference ranges strengthen this suspicion,[22] but it should be noted that the real alcohol consumption of the patient is unknown, as case history was not available. At two occasions, haptoglobin analyses were included, and both results were reported as below the limit of quantification. Reticulocyte values were above the reference range in all analysed samples, and the two measured HbA1c values were below the reference range. In conclusion, these results indicate a possible haemolytic condition in this patient, which could mask a high alcohol consumption when relying solely on PEth measurements.

The present study has some obvious weaknesses, the most important being lack of clinical information about the patients and the fact that all analyses was not performed in all samples. Unfortunately, self-reported information of alcohol consumption was not available. All patients with a combination of low PEth and high CDT are not necessarily prone to falsely low PEth values. In a few, rare cases, PEth values could be true negatives

despite high CDT values. CDT is an indirect alcohol marker, and although it shows a high specificity, it could occasionally be increased due to other conditions than high alcohol consumption, for instance, end-stage liver disease. There also exist genetic conditions and transferrin variants that could cause falsely high CDT measurements[23–25]; unfortunately, these were not tested in the present study. The levels of PEth and CDT could not be directly compared, as elimination occurs at different rates and different consumption patterns therefore could affect the ratio. On the other hand, it is difficult to imagine how these differences between PEth and CDT should be related to the levels of haptoglobin, HbA1c and Hb. Among other weaknesses in the present study, we must emphasise that the study group from patients with possible haemolysis is much smaller than the control group consisting of the rest of the patient samples. Therefore, results should be interpreted with caution.

The strength of the present study is the large study sample size comprising 9893 serum and whole blood samples collected from 5746 patients, analysed in the same laboratory equal for all patients using fully validated, robust analytical methods and consensus cut-off levels.[26]

Although the present study yields no definite answer, we conclude that haemolytic conditions could possibly cause falsely lowered PEth values. Therefore, supplementing PEth with other analyses, such as CDT and diagnostic workup of a possible haemolysis, should be considered if a high alcohol consumption is suspected regardless of low PEth values.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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